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Image contrast immersion method for measuring ...

Block 13: Supplementary Note

Image contrast immersion method for measuring refractive index applied to spider silks

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References and links

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1. Introduction

The motivation to make more accurate measurements of the optical properties of natural spider silks comes from a new appreciation that the aerial webs of certain orb web spiders are as much an optical device, as a mechanical one. The extraordinary mechanical properties of spider silk, dragline silk in particular, are well documented [1–3], but there is relatively little knowledge of the optical characteristics. Dragline silk is differentiated from radial silk by the speed at which the silk is spun – ~10 cm/sec as compared to 1-2 cm/sec for radial silk. Several genera within the family ***Araneidae*** weave orb webs that are highly transparent and have low visibility in most natural lighting conditions. The silks from such webs have been measured to have low UV reflectivity [4]. We have shown that species from the genus ***Argiope*** have silks with high optical surface quality [5,6] and that all components of the web have optical functionality [7]. To better understand the spider orb web as an optical device it is necessary to have accurate knowledge of its bulk optical material properties. Here-in we address obtaining a twenty times improvement in accuracy for the measurement of the refractive index, $\boldsymbol{n}$, of dragline and radial silks of ***Plebs eburnus***. This spider weaves a highly transparent, low visibility orb web.

Refractometry is the most widely used technique for accurately measuring $\boldsymbol{n}$ for transparent media. It has been in use for more than a century. There are several standard refractometry methods [8]. Most require a bulk sample with surfaces polished to optical flatness. Irregularly shaped objects are less suited to standard methods. Liu [9] devised a method for measuring the refractive index of an optical fiber which was based on the immersion of a section of optical fiber in a liquid of known refractive index and observing the refraction of light through the immersed fiber. The refractive index of the cladding layer was evaluated by thermally tuning the refractive index of the liquid and observing the point at which the cladding/liquid interface was rendered invisible.

Another commonly used immersion technique is the Becke line method [10]. When an object, typically a mineral, is immersed in a liquid a bright fringe called a Becke line appears at the object interface. The motion of the fringe as the object is passed through focus, is determined by whether the refractive index of the object is greater or less than the liquid. By using a succession of liquids with different refractive indices, the refractive index of the sample can be deduced. The accuracy of the Becke line method is limited by the visibility of the Becke line. Factors such as object roughness, object inhomogeneity and small object size diminish the visibility of the Becke line and the accuracy of the technique.

Spider silks have previously been studied using scattering methods, however these approaches suffer from limited accuracy due to the small size of the silks [11–14]. In this paper, we present an approach for measuring the refractive index of spider silks that does not suffer from the same accuracy limitations. The basic principle is the same as Liu’s method, however rather than use the same liquid at different temperatures to change the refractive index we use different liquids at the same temperature, so to avoid heating the spider silk during the measurement as its thermo-optic characteristics are unknown. We have also used digital imaging to quantitatively evaluate the contrast cast by the silk due to the degree of index-matching between silk and liquid, rather than subjective visual judgment or observation of Becke lines. This allows the refractive index of the silk to be determined to within $\pm 5 \times 10^{-4}$. Refractive index, dispersion and birefringence measurements of ***Plebs eburnus*** radial silks are presented.

2. Experiment

Silk samples were harvested from webs spun by ***P. eburnus*** housed in a spider laboratory. A 35 mm slide frame was contacted to a spider web. After contact external silks were cut. Such a sample has several capture spiral strands crossed orthogonally by radial silks. It is the radial silks that are measured. Care was taken not to stretch the web in this process. Samples were studied 7-28 days after being spun and stored in a humidity-controlled environment.

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For refractive index measurements, a glass slide was fit underneath the 35 mm slide frame, with the thickness of the slide chosen such that a small separation was maintained between the silk and slide surface to prevent contamination and the possible spread of fluid from the adhesive droplets on the adjacent capture silks to the radial silks. The separation was also small enough so that when a droplet of refractive index liquid is applied to the slide, the silk becomes fully immersed. A set of Cargille refractive-index oils (Series A, AA) was used as the immersion liquids. This set of oils had refractive indices from 1.40 to 1.64 at a wavelength of 589 nm and temperature of 25° C, with intervals of 2×10^{-3} and an uncertainty of $\pm 2 \times 10^{-4}$.

Once immersed, silks were imaged using an Olympus IX-81 microscope in bright-field mode under Köhler illumination. Band-pass filters with transmission bandwidths around 1 nm were used to illuminate the sample with different wavelengths. Measurements were made at 450, 486, 540, 589 and 656 nm, though any wavelength can be used in principle provided the digital camera is sufficiently responsive. Measurements at different polarizations were made by aligning a linear polarizer with the transmission axis parallel or perpendicular to the silk length.

The aperture-stop was opened just enough to give uniform background illumination. Further opening of the aperture-stop decreased the contrast of the imaged silk. The focus was then adjusted to achieve maximum contrast. Figure 1. shows how the appearance of the fiber varies as the silk passes through focus (vertical arrow), and the point of maximum contrast (vertical line). The spider silk is slightly tilted. This tilting of the spider silk with respect to the focal plane occurs during sample mounting and does not affect the measurement provided the same maximum contrast cross-section is used for all images taken when using a single refractive-index oil.

Images were acquired using an Olympus DP72 digital SLR camera with a 4140 × 3096 pixel resolution. To assist with analysis, the exposure time was adjusted for each image so that the background intensity was halfway between the minimum and maximum intensity levels. Subsequent immersions were performed by applying droplets on a different section of the same silk fiber. This alleviated the need to wash the silk between immersions, but an additional uncertainty was accrued if the silk was inhomogeneous along its length. A 2-3 cm length of silk was needed to complete a measurement.

Matlab was used to extract the intensity across the maximum contrast cross-section of the silk. Cross-sections were taken over a length of 25-50 pixel-widths to reduce background noise. The length the cross-sections were taken over can be any value, provided it was kept constant throughout each refractive index measurement. Additionally, any combination of RGB channels could be used provided it was kept consistent throughout the measurement. The contrast of the silk was quantified as the difference between the background grayscale intensity level and the minimum grayscale intensity level. Provided the illumination conditions are not changed during measurement, no further normalization is needed. Figure 2. shows a typical intensity profile and accompanying calculation of the contrast. A sign convention was adopted whereby the contrast was taken to be positive when the silk was defocused toward the objective (i.e. $\boldsymbol{n_{oil} < n_{silk}}$) and negative otherwise (i.e. $\boldsymbol{n_{oil} > n_{silk}}$).

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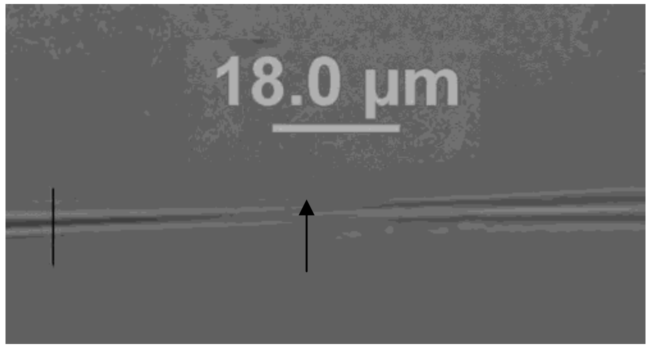

Fig. 1. Immersed spider silk passing through the focal plane along its length due to sample tilt. Note the inversion of the intensity profile as the silk passes through the focal plane (marked by a vertical arrow). The black vertical line indicates the point of maximum contrast used to calculate the visibility of the silk.

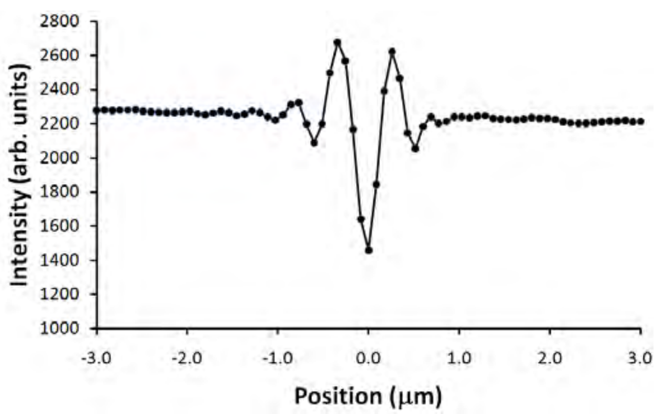

Fig. 2. Intensity profile of the immersed silk shown in Fig. 1 along the point of maximum contrast.

Interpolation between refractive index liquids was done by plotting the contrast as a function of refractive index and using a straight-line fit to determine the point at which the visibility becomes zero. This value is then taken to be the refractive index of the silk. Note that the relationship between refractive index of the oil and the visibility of the immersed silk is only linear when the refractive index mismatch between the silk and oil is small. This difference is less than 6-10 $\times$ 10^{-3} for most of these experiments and depends on the illumination conditions and the exposure settings of the camera. Only oils within this range are therefore utilized when performing linear fits to obtain refractive index measurements.

This approach has several advantages over the Becke line method. Firstly, the contrast (or shadow) is cast by the silk is monitored as it is more visible than the Becke lines, which allows a more accurate measurement of the refractive index of the silk. Moreover, the silk contrast is less affected by factors such as silk roughness, homogeneity and size. Secondly, quantifying the contrast, rather than observing the motion of the Becke line allows interpolation between refractive index oils, further enhancing accuracy. While some interpolation is possible using the Becke line technique through dispersion staining [15], it still requires subjective judgment on the part of the experimentalist.

3. Results

3.1 Optical fiber

This technique was tested on a silica-clad optical fiber (Corning SMF-28) stripped of its acrylite jacket, to verify its accuracy. Optical fiber makes an ideal test subject as the refractive

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index of the fused silica cladding is well known and is exceptionally uniform along its length. Measurements were performed at a wavelength of 589 nm with unpolarized light. Note that the refractive index of the oils used must be temperature-corrected due to the high thermo-optic coefficients of these liquids. Two experimental runs were done in total; for run A, the ambient temperature was 24°C, while for run B, it was 19°C. Also note that due to the larger size of the optical fiber, contrast varies linearly over a much larger refractive index range, and so the choice of oils was adjusted accordingly.

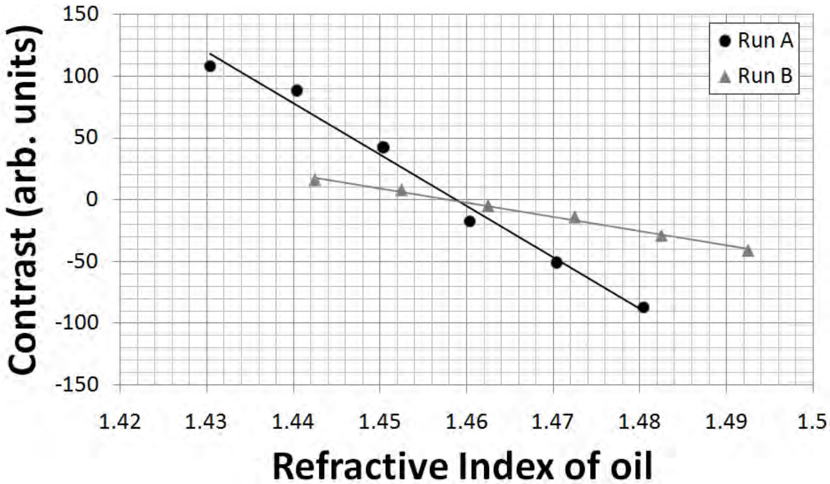

Fig. 3. Measured contrast as a function of the temperature-corrected refractive index of the oil for a Corning SMF-28 optical fiber.

Figure 3. shows the measured contrast as a function of refractive index of the immersion liquid and the linear fits performed for each set of data. The different gradients are due to adjustment of the aperture stop and camera exposure time. The measured refractive index of the silica cladding was the value at which zero contrast was obtained (the point at which the linear fit crosses the horizontal axis). The refractive index of fused silica is 1.45846 at 589 nm as calculated using Sellmeier data. The measured refractive index of the fused silica cladding was measured to be 1.45876 ± 0.00022 and 1.45822 ± 0.00022 for run A and B respectively, giving deviations of (3.0 ± 2.2) × 10^{-4} and (2.5 ± 2.2) × 10^{-4} from the known value. This result demonstrates the ability of this technique to measure refractive index to within an accuracy of ± 5 × 10^{-4}. The uncertainty comes from uncertainty in the refractive index of the liquid and the uncertainty in the temperature of the liquid (due to the high thermo-optic coefficient).

3.2 Spider silk

Refractive index measurements were done for a number of radial silks from webs made by a number of different individual spiders. Measurements were made at 589 nm with the polarization either parallel (denoted $\boldsymbol{n_p}$) or perpendicular (denoted $\boldsymbol{n_s}$) to the length of the silk. The birefringence of the spider silks, $\Delta n = \boldsymbol{n_p}$-$\boldsymbol{n_s}$ was also calculated. Figure 4. shows an example refractive index measurement for a ***P. eburnus*** radial silk (Web 2, Silk A in Table 1 below). The measured values for $\boldsymbol{n_p}$ and $\boldsymbol{n_s}$ were found by performing a linear regression and finding the point at which the linear fit crossed the x-axis. Note that for the two silks in web 1, data was only taken for one polarization which prevented the birefringence from being calculated, however the data is included for comparison.

Refractive index measurements are summarized in Table 1. The uncertainty of some measurements exceeds ± 5 × 10^{-4} because the silks were slightly inhomogeneous along their length, which diminished the quality of the linear fits. Uncertainties were calculated by taking the error in the x intercept (i.e. the predicted value of ***n*** where the contrast is zero) returned by the linear regression analysis and combining it with the uncertainty of ± 5 × 10^{-4} calculated in section 3.1. that arises due to the experimental procedure; and includes uncertainty in temperature and uncertainty in the refractive index of the oil.

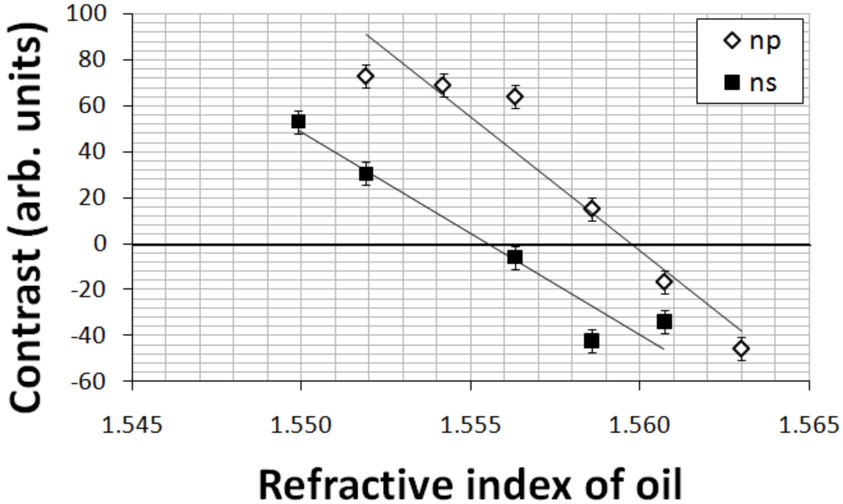

Fig. 4. Measured contrast as a function of the temperature-corrected refractive index of the oil for a ***P. eburnus*** radial silk (Web 2, Silk A in Table 1). The missing point around 1.554 for $\boldsymbol{n_s}$ is due to the presence of an outlier that was omitted prior to performing the analysis.

Table 1. Measured Refractive Indices of *Plebs eburnus* Radial Silks

Web	Silk	n_p	n_s	Δn
1	A	1.5430 ± 0.0011	-	-
1	B	-	1.5438 ± 0.0008	-
2	A	1.5596 ± 0.0008	1.5555 ± 0.0007	0.0041 ± 0.0011
2	B	1.5509 ± 0.0005	1.5511 ± 0.0005	−0.0002 ± 0.0007
3	A	1.5370 ± 0.0013	1.5370 ± 0.0008	0.0000 ± 0.0015
4	A	1.5587 ± 0.0005	1.5458 ± 0.0005	0.0129 ± 0.0007

There are several points worth noting from the measurements presented in Table 1. Firstly, it is assumed the birefringence is primarily uniaxial based on previous measurements and what is known about the structure of the silks themselves [13,16]. Secondly, not all radial silks from different positions in the web are birefringent. The maximum birefringence observed is 0.0129 ± 0.0007. This is less than that measured for silks from the webs of spiders from the genera ***Nephila.*** Values between 0.04 and 0.06 have been reported for the silks of ***N. clavipes*** and all silks measured did show birefringence [16]. This indicates the silk of ***P. eburnus*** may be distinct from that of ***N. clavipes*** with respect to its protein molecular structure. Secondly, there is substantial variation in the refractive index from silks between different webs, up to 0.0226 ± 0.0015 for $\boldsymbol{n_p}$ and 0.0185 ± 0.0011 for $\boldsymbol{n_s}$. This shows that the refractive index, and likely other optical and mechanical properties as well, exhibit variation between individual spiders. Finally, it is worth noting that even in the same web, there is a discernable variability between individual silks, though this variability is smaller than that exhibited from silks from different webs. It is around 0.0087 ± 0.0010 for $\boldsymbol{n_p}$ and 0.0044 ± 0.0009 for $\boldsymbol{n_s}$. It is interesting to speculate whether this variability occurs because of uncontrolled variability in the chemical and physical processes of making the silk or whether there is some design, driven by evolution, behind spinning webs in this manner.

3.3 Dispersion measurements

Dispersion measurements were carried out by performing refractive index measurements at 450 nm, 486 nm, 540 nm, 589 nm and 656 nm and are shown below in Fig. 5. The refractive index of the oils at 450 nm and 540 nm were found using Hartmann's dispersion formula [17].

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The dispersion of the silks was quantified by calculating the Abbe number, ν_D given by;

$$\nu = \frac{\boldsymbol{n_D} - 1}{\boldsymbol{n_F} - \boldsymbol{n_C}}, \tag{1}$$

where $\boldsymbol{n_D}$ is the refractive index at 589 nm, $\boldsymbol{n_F}$ is the refractive index at 486 nm and $\boldsymbol{n_C}$ is the refractive index at 656 nm. The Abbe numbers were calculated to be 31.6 (web 1, silk B) and 32.3 (web 3, silk A). The combination of moderate refractive index and high dispersion exhibited by the radial silks of ***P. eburnus*** is rare among optical materials. It appears in the Abbe diagram at least 0.1 lower in refractive index than any standard glass of similar dispersion.

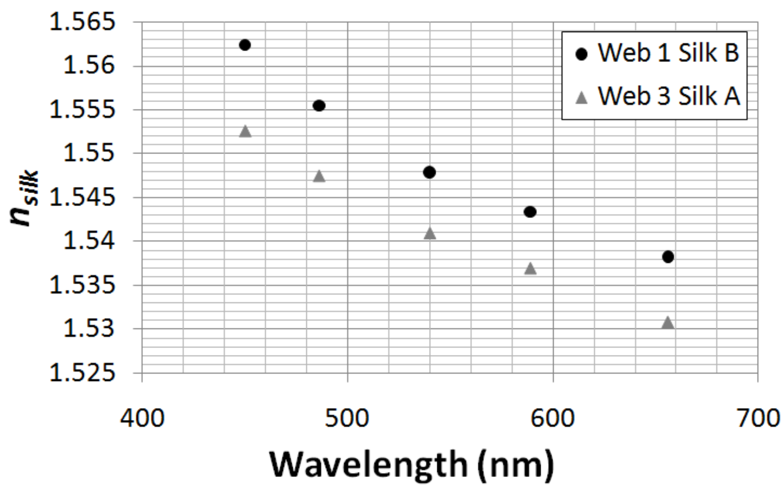

Fig. 5. Refractive index as a function of wavelength for two ***P. eburnus*** radial silks.

4. Conclusion

The refractive index of ***P. eburnus*** radial silks was measured with an accuracy of $\pm 5 \times 10^{-4}$ by immersing the silks in a series of refractive index liquids and measuring the variation in contrast of the silk/oil interface. The measured refractive index for the radial silks ranged from 1.5370 ± 0.0013 to 1.5596 ± 0.0008, demonstrating variability of ~1.5% in the refractive index of the silks. A variability of up to ~0.5% was observed for a single web. Significant birefringence; up to $\Delta \boldsymbol{n} = 0.0129 \pm 0.0007$ was observed for some silks, however some silks exhibited no birefringence at all. The birefringence is less than a third that measured for silks from the spider ***Nephila clavipes*** for which all measured silks had birefringence [16]. This is evidence that the silk of ***P. eburnus*** differs from that of other ***Araneidae*** spiders. The differences have been studied by electron microscopy techniques [18], and are shown to be morphological, at least in part. The dispersion of the silks was also characterized, with Abbe numbers of 31.6 and 32.3 for two individual silks. This demonstrates that ***P. eburnus*** radial silks possess a rare combination of moderate refractive index and high dispersion. These findings are an important discovery toward understanding of the optical properties of spider silks and the possible exploitation of these properties in future bio-inspired materials.

The accuracy of the technique outlined in this paper is limited by the quality of the refractive index liquids, the accuracy to which the temperature of these liquids can be monitored and the quality of the optical system used to image the silks. Improvements to the accuracy of this technique may be possible with further refinements to the experimental setup, such as the use of refractive index liquid sets with intervals less than 0.002. The accuracy of this technique should also be sufficient to detect fine changes in the refractive index as small environmental changes such as temperature or strain are applied to the silk. Therefore, further optical characterization of spider silks such as the measurement of thermo-optic and strain-optic coefficients should be possible using this technique.

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